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Stimulation of mucin secretion from human bronchial epithelial cells by mast cell chymase

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KEY WORDS chymase; mucins; mast cells; epithelial cells; MUC5AC protein

ABSTRACT

AIM: To investigate the effect of chymase on the mucin secretion from human bronchial epithelial cells. **METHODS:** Primarily-cultured human bronchial epithelial (PCHBE) cells and normal human bronchial epithelial (NHBE) cells were cultured with chymase or other stimulus in a mixture of bronchial epithelial growth medium (BEGM) and Dulbecco's modified Eagle's medium (DMEM), and the quantities of stimulatory mucin release were recorded. MUC5AC mucin was measured with an ELISA and dolichos biflorus agglutinin (DBA) mucin was determined with an enzyme linked DBA assay. **RESULTS:** A dose-dependent secretion of DBA mucin from PCHBE cells was observed with chymase with a maximum secretion of 98 % above baseline being achieved following 3 h incubation. The action of chymase started from 1 h, peaked at 3 h and dramatically decreased at 20 h following incubation. Chymase was able to also stimulate approximately 38 % increase in MUC5AC mucin release from PCHBE cells, and about 121 % increase in DBA mucin release from NHBE cells. A chymase inhibitor soybean trypsin inhibitor (SBTI) was able to inhibit up to 85 % chymase induced mucin release, indicating that the enzymatic activity was essential for the actions of chymase on bronchial epithelial cells. **CONCLUSION:** Chymase is a potent stimulus of mucin secretion from human bronchial epithelial cells. It can contribute to mucus hypersecretion process in the patients with chronic obstructive pulmonary disease or asthma.

INTRODUCTION

As a result of airway remodeling with goblet cell metaplasia and submucosal gland hyperplasia, mucus hypersecretion is often a marked feature of patients with asthma[1]. To date, a total of 11 human mucin genes including MUC1-4, MUC5AC, MUC5B, MUC6-8, and MUC11-12 have been identified though the complete cDNA sequences are published only for six mucins MUC1-2, MUC4, MUC5AC, MUC5B, and MUC7[2]. Of these, MUC5AC and MUC5B proteins have been isolated from human airway secretions and are considered to be major constituents of the mucus gel[3,4]. Increased level of MUC5AC protein has been observed in the airways of the subjects with asthma, alone with goblet cell hyperplasia and increased stored mucin, indicating that this type of mucin may contribute to chronic airway narrowing in asthma[5], which suggested that there must be some mechanisms, that is closely involved in the pathogenesis of asthma and can induce MUC5AC mucin hypersecretion in asthmatic airways.

The pivotal role of mast cell in asthma has long been discovered. As the primary effector cell, it is able to induce airway smooth muscle contraction, increased

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vascular permeability, inflammatory cell infiltration and airway remodeling through release of an array of its proinflammatory mediators including tryptase, chymase, carboxypeptidase, histamine, heparin, prostaglandin D_2 (PGD_2), and various cytokines[6]. However, little is known of the role of mast cell in mucus secretion in airways. The observations that level of mast cell tryptase (an indicator of mast cell degranulation) were elevated in the bronchoalveolar lavage fluids from smokers[7] and asthmatics[8], and the numbers of mast cells were increased in the airway epithelium of the subjects with chronic obstructive pulmonary disense (COPD)[9] or asthma[10] implicated that mast cells are involved in the pathogenesis of chronic airway inflammatory disorders. Since hypersecretion of mucus is a hallmark for these diseases mast cells are very likely to play a role in induction of airway mucus secretion. The evidence that dog mast cell chymase is one of the most potent secretagogues for airway gland serous cells[11,12] provides the basis for believing that its human counterpart may have similar effects on mucus secretion cells in human airways. In the current study, the action of mast cell granule product chymase on mucin release from cultured human bronchial epithelial cells was examined and reported herein.

MATERIALS AND METHODS

Materials The following compounds were purchased from Sigma: SBTI, penicillin and streptomycin, tris-base, elastase, S-200 Sephacryl agarose, *N*-succinyl-*L*-Ala-*L*-Ala-*L*-Pro-*L*-Phe-*p*-nitroanilide (SAAPP-*p*-NA), bovine serum albumin (BSA, fraction V), peroxidase conjugated rabbit anti-mouse IgG, biotinylated goat-anti-rabbit IgG, Extr-Avidin peroxidase, bovine pancreas trypsin, 3-(*N*-morpholino)propane-sulphonic acid (MOPS), DBA, peroxidase conjugated DBA, retinoic acid. Foetal calf serum (FCS) and modified M199 medium were from Gibco. Coomassie protein assay reagent was from Pierce (Rockford, IL,USA). A polyclonal antibody against MUC5AC mucin and human mucin standard were provided by Pfizer Limited (Sandwich, UK)[13]. Silver staining kit was from Bio-Rad. BEGM and NHBE cells were from Clonetics (Buckingham, UK). All other chemicals were of analytical grade.

Primary culture of human bronchial epithelial (PCHBE) cells Samples of human bronchial epithelium were obtained from bronchi removed from 8 subjects undergoing lobectomy. In all cases specimens were taken from the bronchi distant from the cancer area and macroscopically normal. Cells in primary cultures were grown from explants of bronchial epithelium that had been microdissected away from underlying connective tissue of bronchial specimens. The explants sized approximately 4-6 mm^2 were plated onto Greiner 24-well tissue culture plates (Greiner) and cultured for 4-6 weeks in a mixture of BEGM and modified M199 medium (1:1, v/v) with 50 nmol/L retinoic acid, at 37 ºC in a humidified incubator (5 % CO_2). During this time, epithelial cells grew to form a confluent monolayer that was approximately 2 cm in diameter around each explant tissue. All cells were grown to around 90 % confluence, before being challenged with chymase or control compounds.

Culture of normal human bronchial epithelial (NHBE) cells NHBE cells were cultured as described previously[13]. Briefly, the cells were seeded in 60-mm tissue culture dishes in BEGM and cultured for 8 d at 37 ºC in a humidified incubator (5 % CO_2). The passage-2 NHBE cells were seeded onto collagen type I gel coated Transwell-clear (Castar Co, Cambridge, USA) culture inserts, and cultured in a mixture of BEGM: Dulbecco's modified Eagle's medium (BEGM:DMEM, 1:1) for 7 d. The air-liquid interface (ALI) was created on d 8, and medium was changed on every other day until cells were challenged.

Stimulation of mucin release from bronchial epithelial cells On the day of challenge, PCHBE cells were first washed twice and then incubated with fresh medium for 1 h, 2 h, 3 h, and 20 h period, respectively before the supernatant being collected for determination of basal mucin release. Similarly, on the d 6 after creation of ALI, NHBE cells were washed and incubated with fresh medium for 2 h and 3 h period, respectively and the supernatants for basal mucin release determination were collected.

Immediately after washing, PCHBE cells were incubated with stimulus or inhibitor in fresh medium for a defined period of 1 h, 2 h, 3 h, or 20 h before supernatant from each well being collected for determination of stimulated mucin release. The challenge for NHBE cells was similar to PCHBE cells, but stimulus or inhibitor were added only into inserts and only for 2 h and 3 h period. All supernatants collected were stored at -20 ºC until use.

ELISA with MUC5AC mucin polyclonal antibody A direct ELISA procedure was developed for mea-

suring human mucin in cell culture supernatants. Mucin standards or samples (50 μL in each well) were coated to a 96-well NUNC Maxisorp plate (Gibco) overnight at 4 ºC. After washing the plate with PBS+0.1 % Tween 20 (PBST), 250 μL of 5 % skimmed milk powder (Marvel) in PBS was added to each well for 60 min at 37 ºC in order to block non-specific protein binding sites. MUC5AC mucin specific antibody (diluted 1:2000 in PBS) was added to the plate and incubated at 37 ºC for 60 min. Biotinylated sheep anti-rabbit IgG (diluted 1:1000) was used as secondary antibody, and this was followed by addition of Extr-Avidin peroxidase (diluted 1:250) into each well. The colour was developed with *o*-phthaldialdehyde (OPD) substrate and the plate was read at 490 nm. The sensitivity of the assay was from 10 μg/L, and interassay variation was less than 5 %.

Enzyme linked lectin assay (ELLA) An ELLA procedure was developed for measuring human mucin in tissue culture supernatants. A 96-well NUNC Maxisorp plate was coated with lectin, dolichos biflorus agglutinin (DBA, 6 mg/L) overnight at 4 ºC, 60 μL each well. After washing with high salt PBS (PBS+0.5 mol/L NaCl+0.1 % Tween 20), 50 μL of mucin sample or standard was added to each well and the plate was incubated at 37 ºC for 40 min. This was followed by addition of peroxidase conjugated DBA (1 mg/L) at 37 ºC for 40 min. The reactions in the plate were visualized by addition of 100 μL OPD substrate and the plate was read at 490 nm. The sensitivity of the assay was from 30 μg/L, and interassay variation was less than 5 %.

Calculation of the mucin released The stimulated (or inhibited) mucin release (SM) was expressed as percentage increase over baseline [% increase=(SM-baseline)/baseline×100 %]. The positive data represent more mucin released over baseline (a stimulatory effect), and negative data mean less mucin released than baseline (an inhibitory effect).

Preparation of chymase Chymase was purified from human skin tissue by high salt extraction, heparin agarose and S-200 sephacryl gelfiltration chromatography procedures as described previously[14]. The purified chymase was then concentrated in C-10 Centricon centrifugal concentrators (Millipore) and stored at -80 ºC until use. Enzymatic activity was determined spectrophotometrically (410 nm) by the rate of hydrolysis of 0.7 mmol/L SAAPP-*p*-NA in 1.5 mol/L NaCl, 0.3 mol/L Tris, pH 8.0. Protein concentration was determined with Coomassie Brilliant Blue G method. The specific activity of chymase was expressed as mU activity per mg protein, where 1 U of enzyme represents that required to hydrolyse 1 μmol of SAAPP-*p*-NA per min at 25 ºC. Purity was evaluated using 10 % SDS-PAGE, and the identity of the protein band confirmed by Western blotting with monoclonal antibody CC1 against human chymase. There was no elaslytical activity and negligible tryptase contamination in this preparation. The specific activity was 4.9 kU/g.

Preparation of compounds As chymase is enzymatically unstable in physiological solutions, considerable care was taken in its preparation. Purified chymase stored in high salt buffer was diluted immediately prior to challenging the cells, first with sterile distilled water adjusting the NaCl concentration to 0.15 mol/L, and then with the culture medium to obtain the required chymase concentration.

Statistics Statistical analysis was performed using SPSS software (version 10.0). Data are shown as the mean±SEM for the number of experiments indicated. Where analysis of variance indicated significant differences between groups, for the preplanned comparisons of interest, paired Student's *t*-test was applied. For all analysis, $P<0.05$ was taken as significant.

RESULTS

DBA mucin release ability of PCHBE and NHBE cells Following a 3-h incubation period, accumulated baseline DBA mucin release from PCHBE or NHBE cells was 507±100 μg/L or 156±13 μg/L, respectively (Tab 1).

Tab 1. Baseline mucin release from PCHBE and NHBE cells following 3 h incubation. *n*=4. Mean±SEM.

Cells	Quantity of mucin released/μg·L^{-1}	
	MUC5AC	DBA
PCHBE	432±130	507±100
NHBE	ND	156±13

PCHBE: primarily-cultured human bronchial epithelial; NHBE: normal human bronchial epithelial; ND: not dertermind.

A dose-dependent release of DBA mucin was observed when PCHBE cells were incubated with mast cell chymase at concentrations of 0.03, 0.3, or 3 mg/L (107 nmol/L) at 37 ºC for 3 h. The maximum induced DBA mucin release was approximately 98 % over

baseline stimulated by 3 mg/L chymase. This was approximately a fold more than that was provoked by neutrophil elastase at concentration of 0.5 mg/L (17 nmol/L) (Fig 1). The time course showed that chymase induced DBA mucin release from PCHBE cells began at 1 h, peaked at 3 h and dramatically declined at 20 h following incubation.

Chymase was also able to induce DBA mucin release from NHBE cells in a dose-dependent manner. The maximum induced DBA mucin release was approximately 121 % over baseline stimulated by 3 mg/L chymase. It appeared that higher concentrations of chymase were required to provoke significant increase in DBA mucin release from NHBE cells than that from PCHBE cells. Trypsin at a concentration of 100 mg/L was able to stimulate up to approximately 225 % increase in DBA mucin release from NHBE cells (Fig 2).

MUC5AC mucin release ability of PCHBE cells After 3 h incubation period, accumulated baseline MUC5AC mucin release from PCHBE cells was 432±130 μg/L (Tab 1).

Mast cell chymase was able to induce a dose-dependent release of MUC5AC mucin from PCHBE cells when the cells were incubated with this serine proteinase at 37 ºC for 3 h. The maximum induced MUC5AC mucin release was approximately 38 % over baseline stimulated by 3 mg/L chymase. This was much less than that was induced by 0.5 mg/L neutrophil elastase (64 %) (Fig 3).

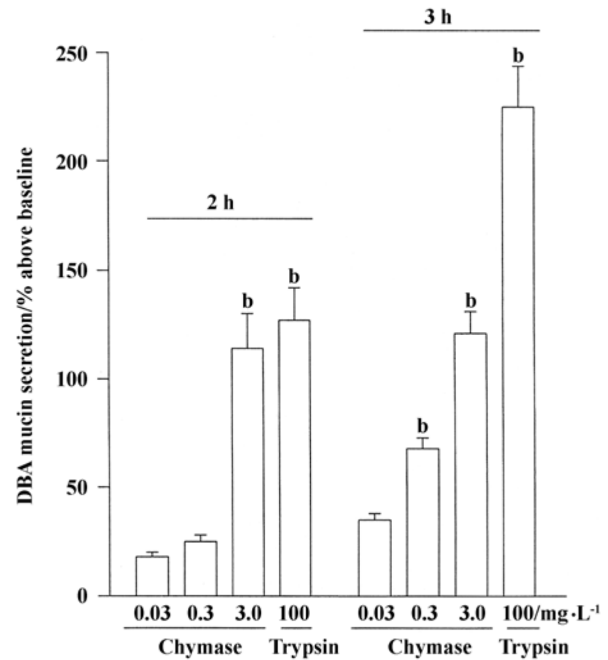

Fig 2. Induction of DBA mucin release from NHBE cells by chymase and trypsin. Cells were incubated with the secretogogues at 37 ºC for 2 h or 3 h. *n*=4. Mean±SEM. [b]*P*<0.05 *vs* baseline control.

Inhibition of chymase induced mucin release by SBTI Chymase (3 mg/L) induced MUC5AC mucin or DBA mucin release from PCHBE cells were reduced by some 82 % and 85 %, respectively by an chymase inhibitor SBTI[15] at 30 mg/L. SBTI was also able to

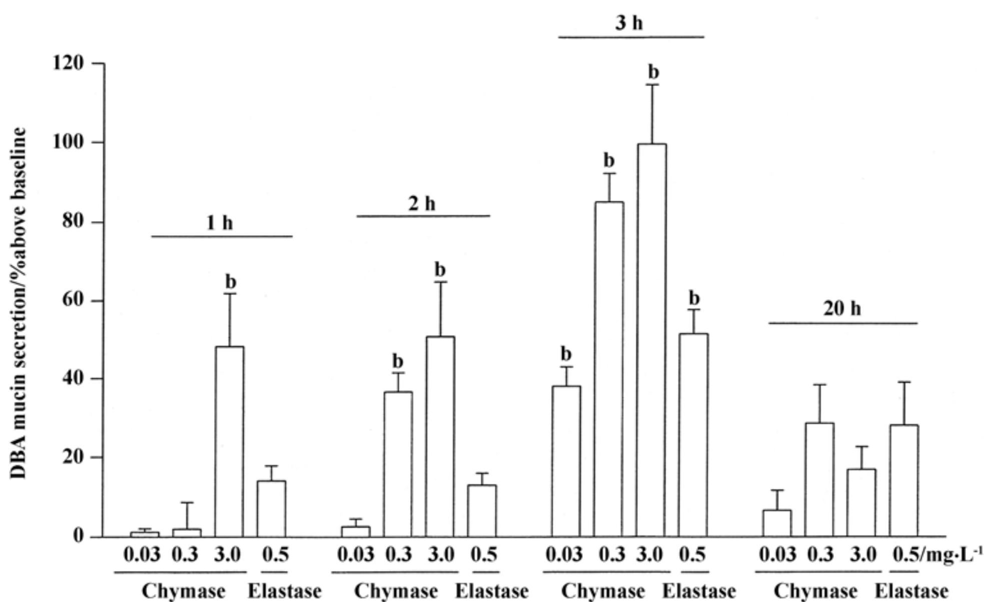

Fig 1. Induction of DBA mucin release from PCHBE cells by chymase and elastase. Cells were incubated with the secretogogues at 37 ºC for various periods. *n*=4. Mean±SEM. [b]*P*<0.05 *vs* baseline control.

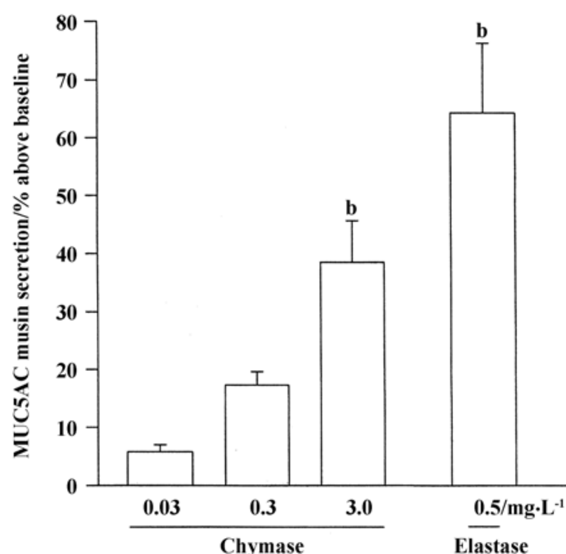

Fig 3. Induction of MUC5AC mucin release from PCHBE cells by chymase and elastase. Cells were incubated with the secretogogues at 37 °C for 3 h. *n*=4. Mean±SEM. [b]*P*<0.05 *vs* baseline control.

inhibit chymase induced DBA mucin release from NHBC cells by approximately 80 % (Tab 2). SBTI by itself did not alter mucin release ability of PCHBE and NHBE cells (data not shown).

Tab 2. Inhibition of chymase (3 mg/L)-induced mucin release by SBTI (30 mg/L). *n*=4. Mean±SEM. [b]*P*<0.05 *vs* uninhibited control.

Cells	Percentage inhibition of mucin release	
	MUC5AC	DBA
PCHBE	82±5[b]	85±6[b]
NHBE	ND	80±5[b]

PCHBE: Primarily-cultured human bronchial epithelial; NHBE: normal human bronchial epithelial; ND: not dertermind.

DISCUSSION

Stimulation of hypersecretion of mucin from bronchial epithelial cells by mast cell granule product chymase proved that mast cell could contribute to mucus overproduction in asthmatic airways upon degranulation, which added a novel evidence for the mechanisms of the mucus blockage or plug formation in the airways of asthma.

Chymase was able to induce approximately 38 % and 98 % increases in MUC5AC and DBA mucin release over baseline from PCHBE, proving that it is a potent stimulus for mucin release in human airways. However, neutrophil elastase appeared a more potent stimulus of mucin release than chymase on equal concentration basis. Investigation on mucus secretion induced by serine proteases, particularly neutrophil elastase and cathepsin G[16] has been carried out for longer than a decade. At present, it is believed that neutrophil elastase is one of the most potent mucus secretagogues in human airways[17]. The action of chymase on mucin release from bronchial epithelial cells was inhibited by an inhibitor of chymase SBTI, indicating that the process was not cytotoxic and required an intact catalytic site of chymase. It was interesting to learn that trypsin was a potent secretagogue of mucin release in human airways, but the mechanism through which this proteinase works requires more investigation.

The most likely explanation for the declined release of mucin induced by chymase at 20 h was chymase is an unstable enzyme, which would certainly lose its all enzymatic activity during 20 h incubation period. At meantime, spontaneous mucin release from the cells would continue, resulting in eventually no significant difference between chymase induced and baseline mucin release from these cells. It was reported that MUC5AC mucin was confined to goblet cells[18,19], whereas DBA was observed to specifically recognize *N*-acetylgalactosamine[20], one of the linking sugars of airway mucin[21]. The observation in the current study that chymase was able to induce both MUC5AC mucin and DBA mucin secretion from bronchial epithelial cells indicated that these two type of mucins at least partially from the same source, airway goblet cells.

The concentrations of chymase employed in the current study should be achieved in the airways of the patients with asthma and COPD as the numbers of mast cells were increased in these pathological conditions and each mast cell containing as much as up to 5 pg chymase[22]. It is worthwhile to point out that retinoic acid was a crucial driving force for transforming normal type of bronchial epithelial cells to mucus hypersecretion type. This was reported previously by Yoon *et al*[23] and Norford *et al*[24].

In conclusion, the mucus hypersecretion is emerging to be an attractive area in COPD and asthma research. Discovery of the important roles of mast cell product chymase in induction of airway mucus secretion would

be beneficial for understanding the pathogenesis of COPD and asthma.

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